

RESEARCH PAPER

Induced production of antifungal naphthoquinones in the pitchers of the carnivorous plant *Nepenthes khasiana*

Haviva Eilenberg¹, Smadar Pnini-Cohen¹, Yocheved Rahamim², Edward Sionov³, Esther Segal³, Shmuel Carmeli² and Aviah Zilberstein^{1,*}

¹ Department of Plant Sciences, The George S Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

² School of Chemistry, The Raymond & Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

³ Department of Human Microbiology, The Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel

* To whom correspondence should be addressed: E-mail: aviah@post.tau.ac.il

Received 4 August 2009; Revised 21 October 2009; Accepted 16 November 2009

Abstract

Nepenthes spp. are carnivorous plants that have developed insect capturing traps, evolved by specific modification of the leaf tips, and are able to utilize insect degradation products as nutritional precursors. A chitin-induced antifungal ability, based on the production and secretion to the trap liquid of droserone and 5-O-methyldroserone, is described here. Such specific secretion uniquely occurred when chitin injection was used as the eliciting agent and probably reflects a certain kind of defence mechanism that has been evolved for protecting the carnivory-based provision of nutritional precursors. The pitcher liquid containing droserone and 5-O-methyldroserone at 3:1 or 4:1 molar ratio, as well as the purified naphthoquinones, exerted an antifungal effect on a wide range of plant and human fungal pathogens. When tested against *Candida* and *Aspergillus* spp., the concentrations required for achieving inhibitory and fungicidal effects were significantly lower than those causing cytotoxicity in cells of the human embryonic kidney cell line, 293T. These naturally secreted 1,4-naphthoquinone derivatives, that are assumed to act via semiquinone enhancement of free radical production, may offer a new lead to develop alternative antifungal drugs with reduced selectable pressure for potentially evolved resistance.

Key words: Antifungal, *Aspergillus*, *Candida*, chitin, droserone, naphthoquinone, *Nepenthes*, pitcher, plant pathogen.

Introduction

Carnivorous plants have evolved special mechanisms for trapping insects and consuming their components when grown under harsh conditions (Ellison and Gotelli, 2009). Carnivory is also characterized by the synthesis of secondary metabolites in the insect-trap tissues, which are used for self-defence (Rischer *et al.*, 2002). The synthesis of protecting secondary metabolites is very common in many plants and occurs in response to chemical, biotic or physical stress (Bringmann and Feineis, 2001; Rischer *et al.*, 2002). Diverse bioactive allelochemicals, including alkaloids, terpenes, and phenolics were found in many representatives of different plant families. These secondary metabolites often possess antiparasitic and pesticidal activities and are considered as potential pharmacological substances and insecticidal leads (Bringmann *et al.*, 1997; Mehmood *et al.*,

1999; Kayser *et al.*, 2003; Khambay *et al.*, 2003; Babula *et al.*, 2009).

Anti-fungal chemotherapy is in constant need of new and effective compounds due to the variable efficacy and adverse effects of the drugs in current use. Furthermore, the intensive use of a limited number of anti-fungal medications has led to the rapid evolution of pathogen resistance and even cross resistance when drugs with closely related modes of actions are in use (Kayser *et al.*, 2003). Considering the limited number of the antifungal agents presently available, characterization of additional sources with different modes of action is of pivotal importance (Akins, 2005).

Naphthoquinones, considered to be potential antifungal drugs, are produced by many plants that belong to the

Caryophyllales families (Rischer *et al.*, 2002), including Nepenthaceae (Bringmann *et al.*, 2000; Aung *et al.*, 2002; Rischer *et al.*, 2002), Droseraceae (Didry *et al.*, 1998; Budzianowski, 2000), Plumbaginaceae (de Paiva *et al.*, 2003), Drosophyllaceae (Bringmann *et al.*, 1998), and Ebenaceae (Dzoyem *et al.*, 2007). Naphthoquinones are synthesized by the shikimate and acetate-malonate (polyketide) pathways (Babula *et al.*, 2009). In the polyketide pathway, condensation of acetyl-CoA with malonyl-CoA yields acetoacetyl-CoA, which is followed by a number of decarboxylative condensations with malonyl-CoA, yielding an oligopolyketide (Pintea, 2007). The acetogenic (derived from acetyl-CoA) origin of droserone (3,5-dihydroxy-2-methyl-1,4-naphthoquinone) was demonstrated in cell cultures of *Triphyophyllum peltatum* (Dioncophyllaceae) (Bringmann *et al.*, 2000). In the Dioncophyllaceae and Ancistrocladaceae (Bringmann *et al.*, 1998) families, which are closely related to Nepenthaceae, naphthylisoquinoline alkaloids are produced from naphthalene and isoquinoline precursors, each originating from six acetate units, via a joint polyketide intermediate. However, in these families naphthoquinones are synthesized mainly during stress, when the isoquinoline alkaloid biosynthetic pathway is inhibited (Bringmann and Feineis, 2001). Therefore, when the transamination step, leading to the biosynthesis of the isoquinoline moiety, is blocked by chemical, physical or biotic stress, only naphthoquinones, for example, plumbagin and droserone are formed (Bringmann and Feineis, 2001). In the pitcher plant, *Nepenthes insignis*, feeding experiments with labelled L-alanine showed that intact C₂-units (acetyl-CoA), produced by oxidative deamination and decarboxylation of L-alanine, serves as an intermediate in the synthesis of plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) (Rischer *et al.*, 2002).

Plumbagin is the most efficient secondary metabolite isolated so far from carnivorous plants. This naphthoquinone possesses anti-malarial (Likhitwitayawuid *et al.*, 1998), antimicrobial (Didry *et al.*, 1998), antifungal (de Paiva *et al.*, 2003; Shin *et al.*, 2007), and anticancer (Parimala and Sachdanandam, 1993; Sandur *et al.*, 2006) activities. However, its high cytotoxicity and relatively low therapeutic selectivity are the major disadvantages that limit its medical application (Kayser *et al.*, 2003). Several plumbagin derivatives have been examined to identify products exerting plumbagin-like fungicidal activity but lower toxicity (Sankaram *et al.*, 1975; Ogihara *et al.*, 1997; Likhitwitayawuid *et al.*, 1998; Hazra *et al.*, 2002; Tandon *et al.*, 2004; Ganapaty *et al.*, 2006). Droserone, the oxygenated derivative of plumbagin, is synthesized in a variety of Nepenthales species (Bringmann *et al.*, 2000; Budzianowski, 2000). It lacks immunostimulating activity (Kreher *et al.*, 1988), but exhibits anti-malarial and possibly pesticidal activity (Bringmann *et al.*, 1993; Bringmann and Feineis, 2001). Other small modifications in the naphthoquinone core structure lead to marked differences in activity and, consequently, also in toxicity. For example, the presence of an oxygen or chlorine atom at the C-3 position of the naphthoquinone nucleus causes weakening or loss of anti-

malarial activity (Likhitwitayawuid *et al.*, 1998). The quinone structure and 5-hydroxyl group of naphthoquinones were both shown to be important for antifeedant activity, serving as a means of chemical defence in the carnivorous plant *Dionaea muscipula* (Tokunaga *et al.*, 2004). Three-methyl-, -ethyl- and -propyl alkylation of 2-hydroxy-1,4-naphthoquinones yielded derivatives that caused both haemolysis and renal damage in rats, although the severity of the effects provoked by these substances was much lower than those induced by the parental compound at an equivalent dose-level (Munday *et al.*, 1995). *In vitro* assays showed that several 2,3-di-substituted 1,4-naphthoquinones are as effective as the clinically used antifungal drugs Fluconazole and Amphotericin-B (Tandon *et al.*, 2008).

Despite the accumulating information about the therapeutic potential of various naphthoquinones, their exact mode of action remains unclear. Two major mechanisms have been proposed for the cytotoxic action of quinones in a variety of biological systems (Inbaraj and Chignell, 2004; Castro *et al.*, 2008). In the first route, quinones undergo one-electron reduction to semiquinone-free radicals by electron-transferring flavin enzymes such as NADPH-cytochrome P450 reductase and mitochondrial NADH-ubiquinone oxidoreductase. These free radicals are oxidized back to quinones by transferring electrons to O₂, generating superoxide anions and hydrogen peroxide. This reduction and oxidation cycle of quinones is defined as 'redox cycling'. It may also lead to glutathione (GSH) oxidation to GSSG by glutathione peroxidase. The second route is based on the action of quinones as potent electrophiles, capable of directly reacting with thiol groups of proteins and glutathione. Interaction with GSH forms mono- and diglutathione-hydroquinone conjugates that can transfer electrons to O₂ and produce hydrogen peroxide and GSSG-quinone conjugates. Redox cycling as well as electrophilic interaction of quinone with glutathione were both shown to be involved in the toxicity exerted by plumbagin and juglone (5-hydroxy-1,4-naphthoquinone) in cultured HaCaT keratinocytes (Inbaraj and Chignell, 2004). While redox cycling was identified as the main mode of action of plumbagin, as deduced by the stoichiometric conversion of GSH to GSSG, the cytotoxicity of juglone could also be attributed to its nucleophilic conjugation with GSH. Similar free-radical-forming mechanisms were also suggested for plumbagin and menadione (2-methyl-1,4-naphthoquinone) in *Saccharomyces cerevisiae* (Castro *et al.*, 2008). Plumbagin and several of its analogues also exert a strong antifeedant activity against *Spodoptera litura* due to a combination of both high volatility and high redox potential (Tokunaga *et al.*, 2004). The identified inhibition of insect ecdysis and chitin synthetase by plumbagin suggests that plumbagin and its closely related derivatives may serve as environmentally friendly insect control agents (Kubo *et al.*, 1983). Plumbagin was also reported to be a potent inhibitor of the NF-kappaB activation pathway, enhancing apoptosis and suppression of cellular proliferation in human cancer cell

lines treated with inflammatory agents (Sandur *et al.*, 2006). Furthermore, many DNA modifications can be attributed to quinonoid compounds, including intercalation, alkylation, induction of strand breaks (nicking), and inhibition of DNA-related enzymes such as topoisomerases (Miguel del Corral *et al.*, 2006).

In the present study, the natural naphthoquinone, droserone and its methylated derivative 2-methyl-3-hydroxy-5-methoxy-1,4-naphthoquinone (5-*O*-methyldroserone, denoted hereafter as Me-droserone), were identified as induced compounds synthesized in closed pitchers of the carnivorous plant *Nepenthes khasiana* following chitin injection. Droserone and Me-droserone, were not present in the closed or open pitchers under normal conditions. The antifungal effect of droserone and Me-droserone was compared. Droserone was more effective, and exerted an antifungal effect on a broad range of human and plant fungal pathogens, when applied as a component of the pitcher liquid or as a purified compound. It is also shown that these naphthoquinones exerted relatively low toxicity to the human cell line, 293T. Considering the wide spectrum of anti-fungal activity and the predicted mode of action that differs from the prevalently used antifungal agents, the chitin-induced pitcher liquid as well as the purified droserone may serve as potential candidates for the development of natural fungicides.

Materials and methods

Biological material

Nepenthes khasiana plants were grown in the Botanic Gardens of Tel Aviv University. Chitin-induced liquid (CIL) was collected from the closed pitchers 5 d after sterile injection of 500 µl (6–9 mg ml⁻¹) colloidal chitin at pH 5.0 (Vessey and Pegg, 1973) into closed pitchers. The colloidal chitin was prepared by grinding commercial Crustacean chitin flakes and subsequent filtering through a 20 mesh nylon membrane. The filtered chitin was washed with boiling water for 1 h, precipitated by centrifugation and the pellet stirred in an ethanol:ether:HCl mix (38:38:0.8 by vol.) for 15 min. Thereafter the mixture was filtered through a gauze and the solid phase solubilized in 0.2 M NaClO for 1 h at 75 °C. After an additional filtering, the solid phase was solubilized by stirring in acetone and 32% HCl (1:150 v:v) at 4 °C. After centrifugation, the supernatant (solubilized chitin) was collected and the chitin was precipitated by adding ice-cold water and incubating at 4 °C overnight. The acid was removed by subsequent washes with cold water until the pH of the supernatant reached 2.5. The chitin paste was solubilized in water by homogenization and the pH of the colloidal chitin was brought to 5 by adding 1 N NaOH. This colloidal chitin was used for the injection. The liquid collected from the pitchers, (denoted as CIL), was lyophilized. The dried material was used for all experiments and termed as dried chitin-induced liquid (DCIL). DCIL was diluted in water and used for all experiments unless otherwise stated.

Prey-induced pitcher liquid was collected from open pitchers containing partly digested natural prey (mainly ants). The prey debris was removed by centrifugation and the supernatant was concentrated to the desired volume by a Speedvac.

Non-induced pitcher liquid was collected from closed pitchers and concentrated by Speedvac to the desired volume.

Purification of 3,5-dihydroxy-2-methyl-1,4-naphthoquinone (droserone) and 2-methyl-3-hydroxy-5-methoxy-1,4-naphthoquinone (5-O-methyldroserone)

DCIL was gel filtrated on a Sephadex LH-20 (Pharmacia) and eluted with methanol/chloroform 1:1 (v:v). The mixture of naphthoquinone derivatives was then flash-chromatographed on a C-18 reversed-phase silica-gel column (YMC-Gel ODS, Irregular 120A, Particle Size 63–210; 10 g silica per 1 g of extract) and eluted with solvents with decreasing polarity (50 ml each): water, 3:1, 1:1, 1:3, and 1:9 water/methanol, methanol, acetonitrile, and ethyl acetate, respectively.

The semipure 5-*O*-methyldroserone and droserone mixture was applied onto a preparative (10 µm, 22×250 mm) RP-18 HPLC column (Alltech, 10 m Econosil or equivalent), eluted with methanol/H₂O (5 ml min⁻¹) and monitored by UV detector at 250 nm. Final purification of 5-*O*-methyldroserone and droserone was achieved by chromatography on a semi-preparative (5 µm, 10×250 mm) HPLC column of RP-18 bonded-silica. Verification of the 5-*O*-methyldroserone and droserone structure was carried out using various spectroscopic and spectrometric techniques (e.g. IR, UV, NMR, and MS).

In vitro antifungal activity against human pathogens

In vitro activities of the tested substances against human fungal pathogens (*Candida* and *Aspergillus* spp.) were determined using the broth micro dilution system with 96 well micro-titration plates according to the CLSI M27-A protocol for yeasts (Wayne, 2002a) and the M38-A protocol for moulds (Wayne, 2002b). Serial dilutions of the substances and the fungi tested were incubated for 48 h at 37 °C. Minimal inhibitory concentration (MIC) of the substances tested was determined spectrophotometrically and defined as the lowest concentration that allows growth similar to that of the negative control (with no fungi). Minimal fungicidal concentration (MFC) was determined by culturing 100 µl of broth from all wells, that showed growth above the MIC, on Sabouraud's dextrose agar (SDA) plates for 48 h at 37 °C. The lowest concentration that killed the fungi was defined as the MFC value.

Germination inhibition of *Mycosphaerella graminicola* (*Septoria tritici*) conidia

Samples of log-phase culture of *M. graminicola* conidia (2.5×10³ conidia/100 µl) were mixed with 100 µl of increasing concentrations of DCIL and incubated in a microtitre plate at 18 °C for 6 d and then the fungistatic effect was determined by measuring absorbance at 550 nm. The fungicidal activity was evaluated by further plating a 50 µl sample of each 6-d-culture on malt agar and estimating growth after an additional 6 d. Non-treated cultures exposed to the same conditions served as controls.

Growth inhibition assay of *Rhizoctonia solani* and *Fusarium oxysporum*

The antifungal activity of CIL was assayed using paper discs soaked with either 12 mg DCIL dissolved in 100 µl H₂O or only in 100 µl H₂O that were placed on the surface of malt agar (*R. solani*) or potato dextrose agar (*F. oxysporum*) plates. An agar plug with a surface area of 3×3 mm² was taken from young cultures of *R. solani* or *F. oxysporum* grown on solid medium and placed in the middle of the plate. The plates were incubated for 4 d at 22 °C. The inhibition zones around the paper discs indicated the antifungal activity.

Growth inhibition assay of *Botrytis cinerea* mycelium on tobacco leaves

Equal volumes of non-induced, chitin-induced, and prey-induced pitcher liquid samples were 16.7-fold concentrated by a SpeedVac

and then a 20 µl drop of each concentrated liquid was applied onto detached tobacco leaves. An agar plug (3×3 mm² surface area) containing young mycelium of *B. cinerea* was placed on each drop. The leaves were incubated in a humid chamber at 22 °C for 6 d and then the size of the lesion, caused by mycelium penetration into the leaf tissues, was estimated.

Inhibition of spore germination of Alternaria solani and Botrytis cinerea

Spores of *A. solani* and *B. cinerea* were incubated in potato dextrose broth (PDB) in microtitre plates in the presence of increasing concentrations of purified droserone, Me-droserone or a 3:1 (molar ratio) mixture at 22 °C. Growth inhibition was determined after 24 h incubation by microscopic observation and after 44 h by measuring the density of the germinating spores at 595 nm.

Cytotoxicity to human embryo kidney cells (293T)

Human embryonic kidney cells (293T) were grown at 37 °C under 5% CO₂ in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal calf serum, 4500 mg l⁻¹ glucose, and 2 mM glutamine. Cells were seeded at a density of 8×10³ per well in a 96 well plate and incubated for 24 h. The medium was then removed and the cells were incubated in the presence of 60 µl of increasing concentrations of droserone or 5-*O*-methyldroserone dissolved in sterile water. Cell viability was determined after 48 h by staining with 0.4% (w/v) trypan blue and haemocytometer counting. The effect of each metabolite was determined in two replicates.

Results

Chitin-induced pitcher liquid as an anti-fungal agent

Chitin is a regular component of the insect skeleton and cell wall of many fungi (Patil *et al.*, 2000). To mimic the prey-capturing conditions in the insect trap, soluble chitin was injected into closed young pitchers of *N. khasiana*. Such chitin injection induces *de novo* synthesis of chitinases and a β-1,3-glucanase in the closed traps (Eilenberg *et al.*, 2006; Eilenberg and Zilberstein, 2008) that might act as insect exoskeleton and fungal cell wall hydrolysing agents. To examine the anti-fungal potential of the non-induced and chitin-induced pitcher liquid (CIL) further, samples of non-induced and chitin-induced pitcher liquid were collected, 5 d after chitin injection, and stored after lyophilization. The antifungal capacity against plant-fungal pathogens (Fig. 1) and several common human fungal pathogens (Table 1) was determined.

Chitin-induced pitcher liquid is effective against plant fungal pathogens

Due to the chitin injection, the pitcher liquid became red (Fig. 1A, red dot) indicating a possible synthesis of either pigments or secondary metabolites that differ from those present in non-chitin-induced (Fig. 1A, white dot) and prey-induced liquid (Fig. 1A, blue dot). The inhibitory effect of non-induced, chitin-induced, and prey-induced pitcher liquids on the growth of *B. cinerea* was compared (Fig. 1B). Co-application of each of the different pitcher liquid samples (20 µl of 16.7-fold concentrated) and an agar cube

containing *B. cinerea* mycelia, on excised tobacco leaves demonstrated that only the CIL efficiently inhibited mycelial growth. Neither the non-induced nor the prey-induced pitcher liquid, which was collected from open traps containing partly digested ants, had any inhibitory effect.

The effect of CIL on the mycelial growth of *R. solani* and *F. oxysporum* (Fig. 1C, D, respectively) was examined next. Paper discs soaked with 12 mg DCIL (dried CIL) dissolved in 100 µl H₂O and located at a 4 cm distance from the initial inoculum of young mycelia, strongly inhibited fungal growth compared with the water control. CIL was also shown to have both a fungistatic as well as a fungicidal effect on *M. graminicola*. Spore germination was partly or completely inhibited in the presence of 17.3 mg ml⁻¹ and 34.5 mg ml⁻¹ DCIL, respectively (Fig. 1E). Inhibition of mycelial growth or spore germination of the common plant pathogens *B. cinerea*, *R. solani*, *F. oxysporum*, and *M. graminicola* suggested a general anti-fungal effect (Fig. 1A–E).

Antifungal effect of chitin-induced pitcher liquid on human pathogens

CIL effectively inhibited the growth of *Candida* and *Aspergillus* species, representing major human fungal pathogens (Mandell *et al.*, 2005) and even exerted a fungicidal effect on both of them (Table 1). *C. albicans*-CBS 562 was the most sensitive among the tested yeasts, having the lowest MIC of 2.5 mg ml⁻¹ DCIL. The CIL concentration that inhibited growth of *C. albicans* CBS 562 was about 3-fold and 6-fold lower than that required for growth inhibition of *C. krusei* and *C. glabrata*, respectively. The range of DCIL fungicidal concentrations (MFC) was between 12.5–14.5 mg ml⁻¹ for the different *Candida* species, the lowest still being for *C. albicans*-CBS 562. The growth of all three pathogenic moulds was also inhibited by CIL (Table 1), with *A. flavus* and *A. niger* being the most susceptible and *A. fumigatus* the most resistant species. The MIC and MFC values were identical for *A. flavus* (1.9 mg ml⁻¹) whereas *A. fumigatus* and *A. niger* were killed by 14.5 mg ml⁻¹ and 7.3 mg ml⁻¹ DCIL, respectively, reaching the range required for *Candida* sp. lethality.

The above results indicate that CIL exerted a general antifungal effect causing growth retardation and lethality of various plant and human fungal pathogens. Interestingly, the CIL-antifungal effect was lost after dialysis (pore size MW 10 kDa) of the chitin-induced liquid against H₂O (data not shown), suggesting the involvement of low molecular weight molecules.

Chemical analysis of the active compounds

The presence of the low molecular weight molecule/s was assessed next by NMR analysis of CIL. Samples of concentrated chitin-induced, prey-induced, and non-induced pitcher liquid were separated on a reversed phase column and the eluted fractions were subjected to NMR analysis. Two secondary metabolites, droserone and Me-droserone

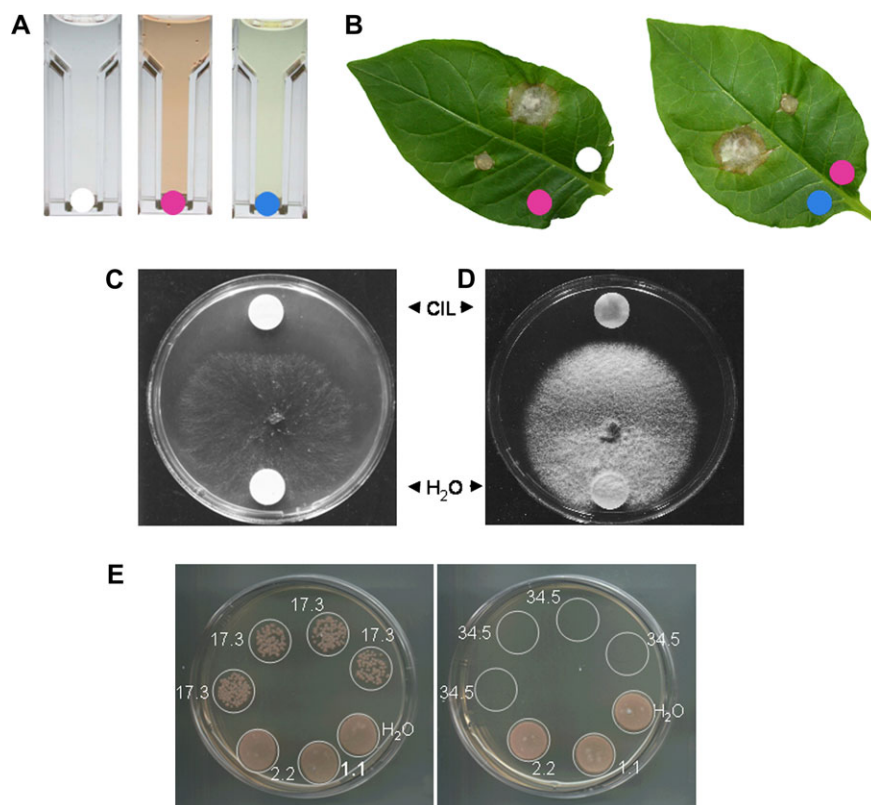


Fig. 1. Antifungal activity of non-induced, chitin-induced, and prey-induced pitcher liquid against plant fungal pathogens. (A) Chitin injection into closed pitchers induced a change in the liquid colour. Comparison of samples of non-induced (white dot), chitin-induced (red dot) and prey-induced (blue dot) pitcher liquid. (B) *B. cinerea* growth inhibition measured on excised tobacco leaves. Samples of 20 µl of 16.7-fold concentrated non-induced, chitin-induced, and prey-induced pitcher liquid were separately applied on the leaves. An agar plug containing *B. cinerea* young mycelium was placed on each spot and the leaves were incubated in a humid growth chamber at 22 °C for 6 d. (C) Inhibition of *R. solani* and (D) *F. oxysporum* mycelial growth. Paper discs were soaked either with 12 mg DCIL/100 µl H₂O or 100 µl H₂O and applied on the surface of malt agar/potato dextrose agar plates. An agar plug (9 mm²) of *R. solani* or *F. oxysporum* young mycelium was placed in the centre. The plates were incubated for 4 d at 22 °C. (E) Inhibition of *M. graminicola* conidia germination. *M. graminicola* conidia (2.5×10^3 conidia in 100 µl) were incubated in microtitre plates in the presence of increasing amounts of DCIL dissolved in 100 µl H₂O at 18 °C for 6 d (the final concentration in mg ml⁻¹ is indicated in E). Thereafter 50 µl of each culture was plated onto a malt agar plate and incubated for an additional 6 d under the same conditions.

Table 1. The effect of chitin-induced pitcher liquid (CIL) on human fungal pathogens

Fungicidal assays were carried out using log phase cultures of the indicated fungal pathogens and various concentrations of the chitin-induced pitcher liquid. Non-induced pitcher liquid was tested against all four *Candida* species and had no antifungal effect even at 57.4 mg ml⁻¹.

Pathogens	MIC ^a (DCIL mg ml ⁻¹)	MFC ^a (DCIL mg ml ⁻¹)
Yeasts		
<i>Candida albicans</i> CBS 562	2.5	12.5
<i>Candida albicans</i> mas	3.5	14.5
<i>Candida krusei</i>	7.3	14.5
<i>Candida glabrata</i>	14.5	14.5
Moulds		
<i>Aspergillus fumigatus</i>	3.8	14.5
<i>Aspergillus flavus</i>	1.9	1.9
<i>Aspergillus niger</i>	1.9	7.3

^a MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; DCIL, dried chitin-induced liquid.

(Fig. 2), were detected in the chitin-induced liquid (Fig. 2B), but not in non-induced or in prey-induced pitcher liquid (Fig. 2A, C, respectively). The NMR analysis of CIL indicated a 4:1 (Fig. 2B) or 3:1 (data not shown) droserone:Me-droserone molar ratio. Consequently, large amounts of CIL were used to purify these naphthoquinones by a preparative reversed phase open column using 70% methanol in water as the eluant. The mixtures of droserone and Me-droserone (3:1 or 4:1 molar ratio) as well as the two pure compounds were used for estimating antifungal activity.

The effect of droserone and Me-droserone mixture on plant- and human-pathogenic fungi

The antifungal effect of the 3:1 mixture of droserone:Me-droserone was examined by incubating increasing concentrations of the mixture with *A. solani* and *B. cinerea* spores (2×10^3) in microtitre plates for 24 h (Fig. 3A, C, respectively). After an additional 20 h incubation, the

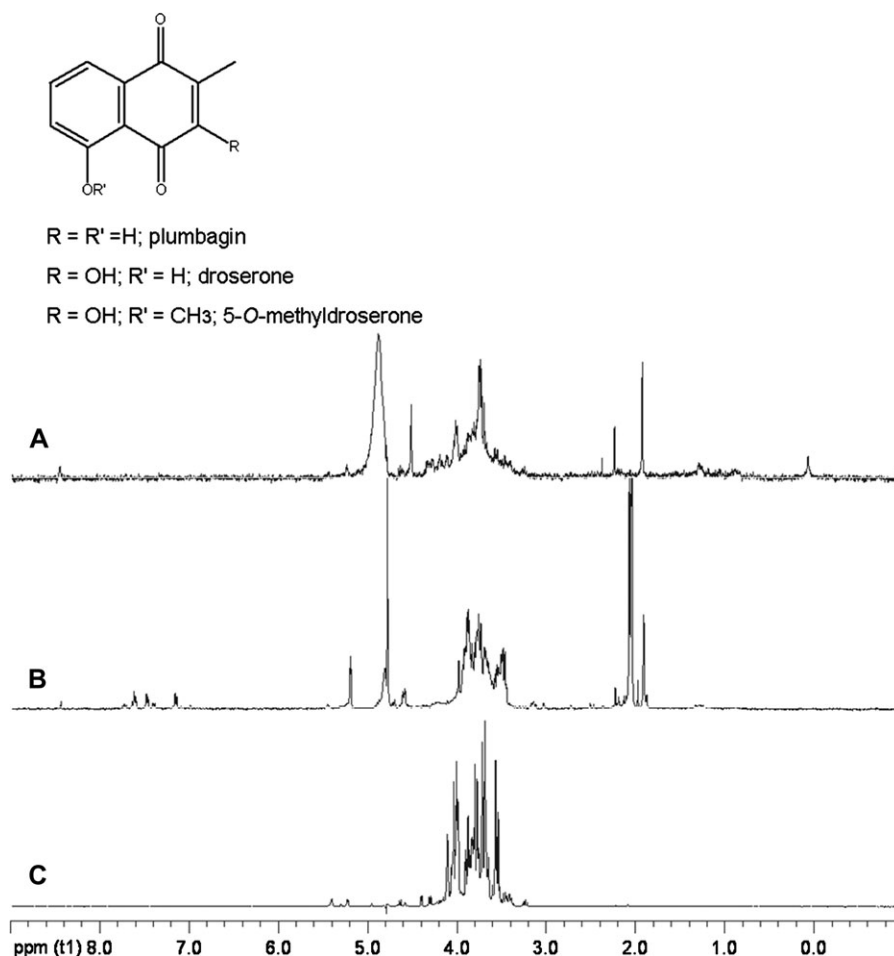


Fig. 2. Identification of droserone and 5-O-methyldroserone by NMR analysis. The structure of compounds (upper panel) and ¹H NMR spectra of (A) non-induced, (B) chitin-induced, and (C) prey-induced (open pitcher) liquid. The proton signals, indicative of droserone and 5-O-methyldroserone, consist of the aromatic proton between 7 ppm and 8 ppm and the methyl protons that appear as singlets between 1.8 ppm and 1.9 ppm. The 5-methoxy group resonates as singlet at 4.0 ppm. A ratio of 4:1 of droserone to 5-O-methyldroserone was observed in the sample of chitin-induced liquid while these metabolites were absent from the non-induced and prey-induced pitcher liquid. The ratio between droserone and 5-O-methyldroserone was determined by measuring the integration of the vinyl methyl at position 2 of the naphthoquinones, which resonate at 1.92 ppm for droserone, and 1.99 ppm for 5-O-methyldroserone.

pathogen viability was determined by measuring absorbance at 595 nm (Fig. 3B, D). At a concentration of 66 µg ml⁻¹, consisting of 49 µg ml⁻¹ and 17 µg ml⁻¹ droserone and Me-droserone, respectively, there was already more than 50% inhibition of *A. solani* spore germination (Fig. 4A, B). For *B. cinerea* the 50% inhibitory concentration was higher, between 66 µg ml⁻¹ to 330 µg ml⁻¹. The latter concentration resulted in almost total inhibition of spore germination (Fig. 4C, D). The fungicidal effect of the mixture was further confirmed after 44 h by plating the cultures from each well on potato dextrose agar (PDA) plates and measuring viability after 5 d. A complete fungicidal effect on both *A. solani* and *B. cinerea* was obtained at 330 µg ml⁻¹ (data not shown).

Next the effect of the 3:1 mixture of droserone:Me-droserone on human pathogens was estimated by determining the MIC and MFC values on three different *Candida* species and *A. fumigatus* (Table 2). Amphotericin B (AMB) (Squibb), whose MIC value is <1 µg ml⁻¹ for *Candida* spp.

(Espinel-Ingroff *et al.*, 1995), served as the control antifungal drug. Table 2 demonstrates that the droserone/Me-droserone mixture exerted a growth inhibitory effect and fungicidal activity on all the fungi tested. The growth of *Candida* species was more affected than that of the mould *A. fumigatus*, while the fungicidal effect was equal in all the organisms tested. These results indicate that both plant and human fungal pathogens were susceptible to the 3:1 droserone:Me-droserone mixture.

Comparison of the antifungal effect of droserone and Me-droserone

To evaluate the antifungal effect of each of the two compounds, separate assays were carried out with droserone and Me-droserone purified from the CIL. *A. solani* spores were separately incubated in microtitre plates with increasing concentrations of droserone (Fig. 4A) or Me-droserone (Fig. 4B) for 44 h. Droserone, which was also

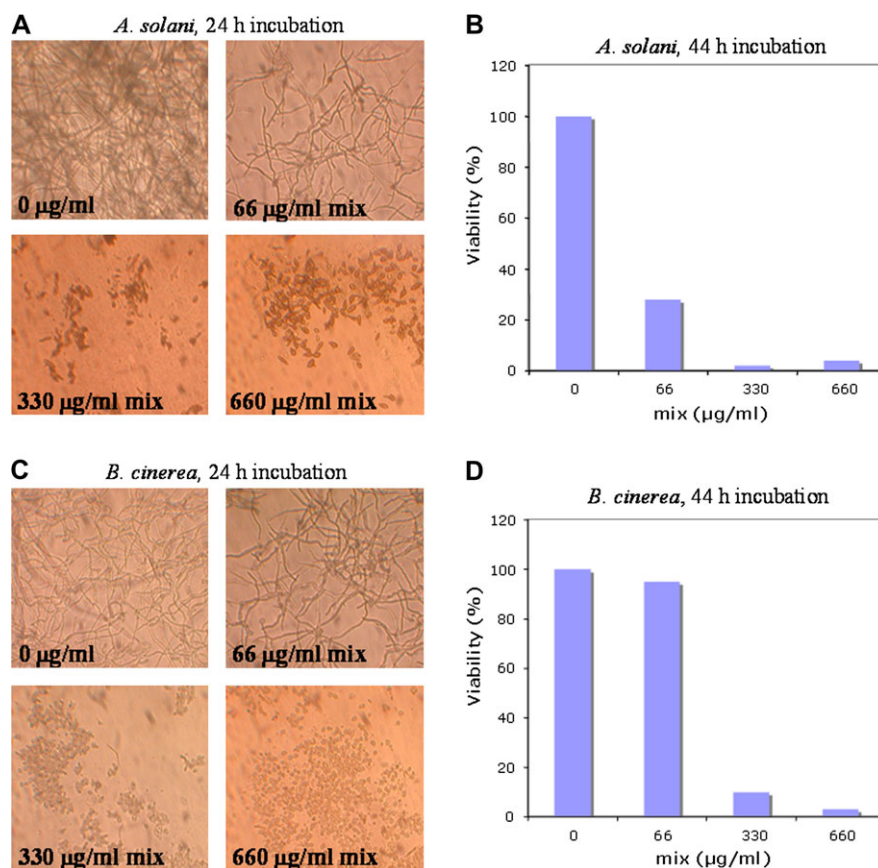


Fig. 3. Inhibition of *A. solani* and *B. cinerea* spore germination by a droserone/Me-droserone mixture (3:1 molar ratio). *A. solani* (A, B) and *B. cinerea* (C, D) spores (2×10^3) were incubated in PDB in microtitre wells in the presence of increasing concentrations of droserone/Me-droserone mixture (3:1 molar ratio). The droserone concentration was 49, 243, and $486 \mu\text{g ml}^{-1}$ in the 66, 330, and 660 $\mu\text{g ml}^{-1}$ mixtures, respectively. Inhibition of spore germination was determined after 24 h by microscopic photographing (A, C) and after 44 h by measuring the density of germinating spores at 595 nm (B, D).

found to be responsible for the red colour of the CIL (Fig. 1A), was far more effective than Me-droserone, reaching a complete inhibition of growth at $163 \mu\text{g ml}^{-1}$ (0.8 mM) (Fig. 4A), whereas Me-droserone only exerted a partial inhibitory effect at $650 \mu\text{g ml}^{-1}$ (3 mM) (Fig. 4B). These data demonstrated that droserone is the more effective fungicide. Thus, in the 3:1 mixture, the antifungal effect could be attributed to droserone rather than to Me-droserone.

Cytotoxicity of droserone and Me-droserone on human cells

To assess the cytotoxic effect, cells of the 293T line, derived from a human embryonic kidney, were exposed to increasing concentrations of purified droserone and Me-droserone. Cell viability was determined after 48 h incubation. Both droserone and Me-droserone caused an approximately 15% reduction in cell viability in all tested concentrations up to $650 \mu\text{g ml}^{-1}$. A higher cytotoxic effect of droserone compared to Me-droserone was observed only at $1300 \mu\text{g ml}^{-1}$ (6.4 mM and 6.0 mM, respectively). At this Me-droserone concentration, 42% of the cells were still viable, while 7% of the cells were alive in the presence of droserone (Fig. 5),

indicating that, at high concentrations, Me-droserone is less toxic than droserone to human cells. However, the cytotoxic effect of droserone on human cells is many fold lower than its effective concentrations against human fungal pathogens (Fig. 5; Table 2). According to Table 2, the droserone concentration in the mixture required to achieve a 50% MIC value was $46 \mu\text{g ml}^{-1}$ for *C. albicans* and *C. krusei* and $92 \mu\text{g ml}^{-1}$ or $184 \mu\text{g ml}^{-1}$ for *C. glabrata* or *A. fumigatus*, respectively. These values are significantly lower compared with those causing significant cytotoxicity in human cells ($>650 \mu\text{g ml}^{-1}$) (Fig. 5).

Discussion

Several hydrolytic enzymes, such as proteases, chitinases, and a β -1,3-glucanase were identified in the pitcher liquid of *Nepenthes* spp., providing a digestive ability to the insect trap (Gallie and Chang, 1997; An *et al.*, 2002; Eilenberg *et al.*, 2006; Eilenberg and Zilberstein, 2008). However, no secondary metabolites with antifungal or antibacterial properties were identified in the pitcher liquid despite the anthropological evidence of using the liquid from unopened traps as a curing means, for example, as laxatives and treatments of burns and skin disorders (Etkin, 2008). By

contrast, several naphthoquinone derivatives were identified as secondary metabolites produced in other tissues of *Nepenthes* spp., including leaves (*N. gracilis*), roots (*N. rafflesiana* and *N. thorelii*) and whole-plant extracts (*N. insignis*) (Bringmann et al., 2000; Aung et al., 2002; Rischer et al., 2002). Considering the pitcher liquid as a niche enriched in organic material, it is speculated that a certain pitcher anti-fungal mechanism should have evolved to avoid the fast consumption of organic compounds by competitors before being absorbed by the pitcher cells.

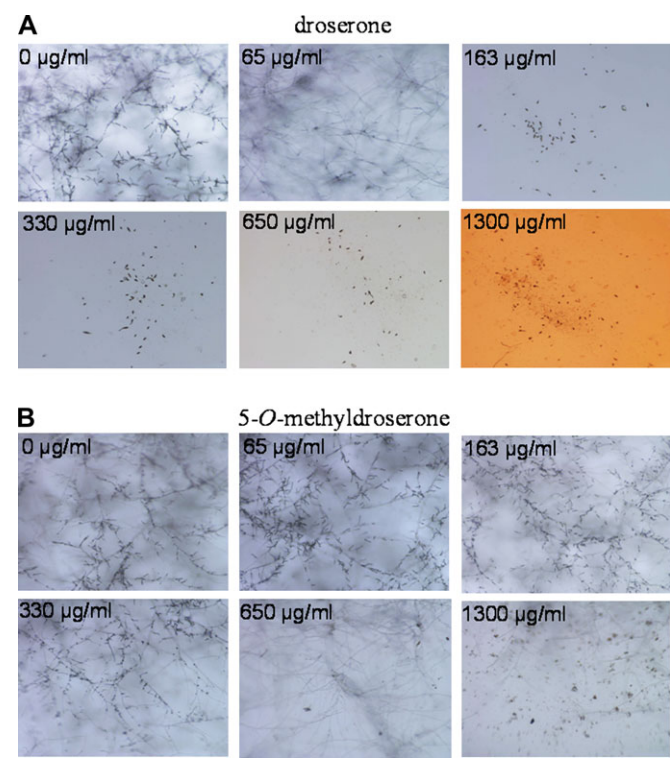


Fig. 4. Inhibition of *A. solani* spore germination by purified droserone and Me-droserone. *A. solani* spores (2×10^3) were incubated in PDB in microtiter wells in the presence of increasing concentrations of droserone (A) or Me-droserone (B). Inhibition of spore germination was determined after 48 h by microscopic photographing.

This study estimated the antifungal capacity of *N. khasiana* pitcher liquid. Chitin was used as an inducing tool in an attempt to mimic natural carnivorous circumstances, since it is the major exo- and endoskeletal component of insect preys. The antifungal activity of liquid collected from chitin-injected closed young traps was compared with liquid samples collected from prey-challenged-open traps and closed young traps. Only the chitin-induced liquid efficiently inhibited human and plant fungal pathogens (Fig. 1; Table 1), indicating that either chitin itself or its breakdown products induced the synthesis of antifungal agent/s, that are not produced under natural conditions either in closed or in open pitchers. Chitin induction not only elicited the production of anti-fungal compounds but also induced the synthesis of endochitinase isoenzymes, with substrate specificity for long chitin polymers (Eilenberg et al., 2006). In other plants, small chitin polymers act as potent elicitors of a variety of defence-related activities, for example, increased lignification in wheat leaves (Barber et al., 1989), induced β -1,3 glucanase expression in cultured barley cells (Kaku et al., 1997), induced phytoalexin synthesis (Yamada et al., 1993), generation of reactive oxygen species (Kuchitsu et al., 1995), and induced expression of defence-related genes in cultured rice cells (Nishizawa et al., 1999; Takai et al., 2001). Different elicitors derived from fungal, bacterial or yeast cell wall extracts were used to increase the accumulation of specific metabolites in various plant cell cultures (DiCosmo and Misawa, 1985; Ramakrishna et al., 1993; Rajendran et al., 1994). For example, chitosan or chitin could enhance plumbagin production and secretion in *Plumbago rosea* suspension culture (Komaraiah et al., 2002, 2003) or in suspension cultures of *Drosophyllum lusitanicum* (Nahalka et al., 1998), respectively. It is likely that the chitin-induced response of pitchers has evolved from an induced defence mechanism that allowed the synthesis of protective compounds only upon sensing the presence of other organisms. This is especially important for carnivorous plants that grow mostly in nutrient-poor habitats where energy and nutritional sources are limited. The absence of antifungal activity in the liquid of untreated closed or open pitchers, suggests that a certain threshold of chitin concentration has to be reached for triggering the

Table 2. Susceptibility of human fungal pathogens to the 3:1 droserone:Medroserone mixture measured *in vitro*

Antifungal activity was assayed as described in the Materials and methods. The mixture concentrations tested were 63, 125, and 250 µg ml⁻¹. 50% MIC is the value that allows 50% growth in comparison to the untreated control.

Human pathogens	50% MIC (µg ml ⁻¹)	Concentrations of droserone (dro) and Me-droserone in the mix (µg ml ⁻¹)		MFC (µg ml ⁻¹)
		dro	Me-dro	
<i>Candida albicans</i> CBS 561	63	46	17	250
<i>Candida glabrata</i>	125	92	33	250
<i>Candida krusei</i>	63	46	17	250
<i>Aspergillus fumigatus</i>	250	184	66	250

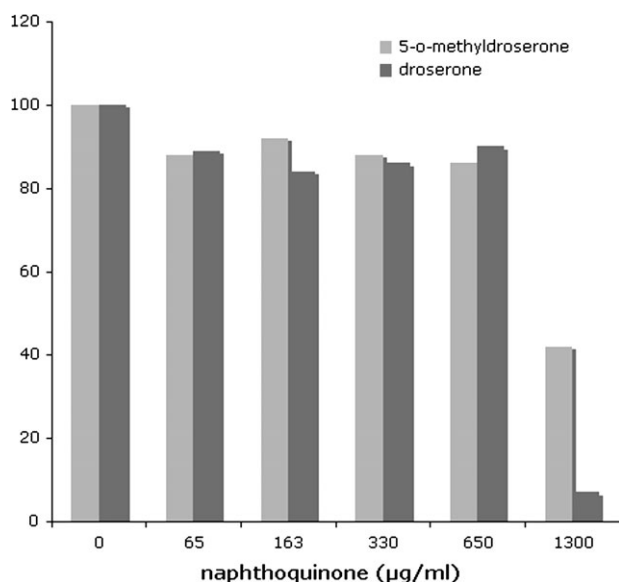


Fig. 5. The effect of droserone and Me-droserone on the viability of human 293T cells. Human-embryo-kidney cells (line 293T) were grown at 37 °C under 5% CO₂ in DMEM containing 10% foetal calf serum, 4500 mg l⁻¹ glucose, and 2 mM glutamine. Cells were seeded at a density of 8 × 10³ per well in a 96-well plate and incubated for 24 h. The medium was then exchanged for media containing 60 µl of increasing concentrations of Me-droserone (light grey) or droserone (dark grey), purified by reversed phase column and identified by NMR. After 48 h incubation, cell viability was determined by staining with 0.4% (w/v) trypan blue. Each treatment contained two replicates.

synthesis of anti-fungal effectors and certain chitinases. As yet, the mechanism underlying the pitcher response to chitin in *N. khasiana* is not clear and may include both the induced synthesis of new compounds and/or the release of existing compounds from the pitcher gland cells to the trap liquid.

The increasing need for new compounds for fungal chemotherapy in medicine has led to high throughput screening of many plant sources for antifungal activity. Alcoholic and aqueous crude extracts of several medicinal plants exhibited antifungal activity against *C. albicans* and the dermatophytes *Epidermophyton floccosum*, *Microsporium gypseum*, and *Trichophyton rubrum* (Mehmood *et al.*, 1999). The stem bark of *Diospyros crassiflora* Hiern (Ebenaceae) showed antifungal activity against several yeast pathogens and filamentous fungi (MIC ranging from 12.5 mg ml⁻¹ to 25 mg ml⁻¹) (Dzoyem *et al.*, 2007). Extracts of *Artemisia annua* and tropical lianas demonstrated high antifungal activity, which was attributed to phenolics, terpenes, and alkaloids (Kayser *et al.*, 2003). Plant-originated naphthoquinones have been widely used against various human tumours and parasitic diseases in some parts of Asia and South America (Etkin, 2008; Babula *et al.*, 2009). Although the curing mechanism of naphthoquinones is not clear, their accompanying cytotoxic effect has been attributed to the 1,4-naphthoquinone nucleus (Miguel del Corral *et al.*, 2006). Additional observed effects of naphthoquinones

include antiviral (Perez-Sacau *et al.*, 2003; Tandon *et al.*, 2004), molluscicidal (Silva *et al.*, 2005), anti-inflammatory, antiplatelet, antiallergic (Lien *et al.*, 1996), antimalarial (Likhitwitayawuid *et al.*, 1998; Biot *et al.*, 2004), antileishmanial (Mantyla *et al.*, 2004), antibacterial, antifungal (Tandon *et al.*, 2004, 2005, 2008), and antiproliferative (Shchekotikhin *et al.*, 2004; Tandon *et al.*, 2004) activities.

In this study it is demonstrated that chitin-induced pitcher liquid of *Nepenthes* exerted both fungistatic and fungicidal activity on several human (Table 1) and plant (Fig. 1) pathogenic fungi, suggesting a broad spectrum of activity. Using NMR analysis it is shown that, in closed *Nepenthes* pitchers, chitin injection induced the production of the naphthoquinones droserone and Me-droserone, which are absent from the non-induced or prey-challenged open pitchers (Fig. 2). Droserone was identified in several plant families, for example, Plumbaginaceae (Dinda *et al.*, 1995), Nepenthaceae (Likhitwitayawuid *et al.*, 1998), Droseraceae (Kreher *et al.*, 1990; Budzianowski, 2000), and Dioncophyllaceae (Bringmann *et al.*, 2000) and was shown to exert antimalarial (Likhitwitayawuid *et al.*, 1998) and pesticidal activities (Bringmann *et al.*, 1993; Bringmann and Feineis, 2001). Me-droserone is rare in nature and was only identified in *Diospyros melanoxylon* Roxb. (Coromandel Ebony Persimmon) (Sidhu *et al.*, 1968).

Droserone was found to be the prominent antifungal agent in the chitin-induced liquid while Me-droserone, the 5-*O*-methylated derivative of droserone, was significantly less effective (Fig. 4). This is in agreement with the findings of Tokunaga *et al.* (2004) that the presence of the 5-hydroxyl group in naphthoquinones is important for chemical defence in the carnivorous plant *Dionaea muscipula*. The droserone/Me-droserone (3:1 molar ratio) mixture was effective against both plant and human fungal pathogens (Fig. 3; Table 2). The concentration required for reaching 50% MIC in all the human pathogens tested was lower than the cytotoxic concentration of each naphthoquinone measured in human embryonic kidney cells (293T cell line; Fig. 5). This relatively low cytotoxicity of droserone and Me-droserone to the human cells is important since plumbagin, considered as a highly effective naphthoquinone, exerts much higher cytotoxicity to human cells. A LD₅₀ value of 3 µM, that was obtained by applying plumbagin to human HeLa cells (Montoya *et al.*, 2005), is about three orders of magnitude lower than the LC₅₀ of 3.2 mM (650 µg ml⁻¹; Fig. 5) observed by applying droserone to 293T cells. Hence, droserone may have an advantage as a candidate for designing drugs for medical applications. Furthermore, the broad spectrum of droserone antifungal activity on diverse fungal pathogens suggests a mechanism that is probably based on the capacity of the naphthoquinone derivatives to generate free radicals and oxidative stress following their reduction by electron-transferring flavin enzymes (Castro *et al.*, 2008). This mechanism may serve as a suitable alternative to those used by present anti-human-fungal-pathogen drugs.

Although new antifungal agents have been introduced during the last decade, therapy of invasive fungal infections

is still problematic (Michallet and Ito, 2009). Thus, the necessity for additional novel antifungal drugs, particularly those acting by different mechanisms than the currently available therapeutic agents, warrants further investigation. The clinically prevalent antifungal drugs, including Amphotericin-B and many azoles, such as fluconazole, act mainly through binding to sterols or inhibiting their biosynthesis in fungi (Akins, 2005). However, their intensive use not only enhances the evolution of drug-resistant mutants in the ergosterol biosynthesis pathway, but also promotes the selection of modified components of the ABC (ATP binding cassette) transporter machinery, including multi-drug transporter genes and related regulatory genes (Coste *et al.*, 2007). For example, since the genes encoding the major components of both mechanisms are localized to chromosome five in *C. albicans*, azole resistance was also evolved as a segmental aneuploidy of this chromosome (Selmecki *et al.*, 2006). Other anti-fungal drugs are in use either alone or in combinations that cover more than a single mode of action. However, broadening the spectrum of antifungal agents and encompassing additional modes of actions are still of the utmost importance (Akins, 2005).

The trend of over-producing secondary metabolites in plant cell cultures has received much attention because intact plants often produce small amounts of the desired metabolites (Komaraiah *et al.*, 2002, 2003). Even in plant cell cultures the yield of secondary metabolites is often low or there is inconsistency in the production and the storage of metabolites within the cellular compartments. Recovery of products from cultures needs harvesting, extraction, and purification, whereas, the induction of naphthoquinone synthesis by chitin injection into *Nepenthes* pitchers represents a convenient method of obtaining pure liquid enriched in droserone *in planta* that can also be used as a promising natural antifungal agent with no additional purification.

Acknowledgements

This study was partially supported by Biotech MAH (Makhteshim-Agan-Hazera) Brurim MP Shikmim 79837, Israel.

References

- Akins RA.** 2005. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. *Medical Mycology* **43**, 285–318.
- An CI, Fukusaki E, Kobayashi A.** 2002. Aspartic proteinases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco. *Planta* **214**, 661–667.
- Aung HH, Chia LS, Goh NK, Chia TF, Ahmed AA, Pare PW, Mabry TJ.** 2002. Phenolic constituents from the leaves of the carnivorous plant *Nepenthes gracilis*. *Fitoterapia* **73**, 445–447.
- Babula P, Adam V, Havel L, Kizek R.** 2009. Noteworthy secondary metabolites naphthoquinones: their occurrence, pharmacological properties and analysis. *Current Pharmaceutical Analysis* **5**, 47–68.
- Barber M, Bertram R, Ride J.** 1989. Chitin oligosaccharides elicit lignification in wounded wheat leaves. *Physiological and Molecular Plant Pathology* **34**, 3–12.
- Biot C, Bauer H, Schirmer R, Davioud-Charvet E.** 2004. 5-Substituted tetrazoles as bioisosteres of carboxylic acids. Bioisosterism and mechanistic studies on glutathione reductase inhibitors as antimalarials. *Journal of Medical Chemistry* **47**, 5972–5983.
- Bringmann G, Feineis D.** 2001. Stress-related polyketide metabolism of Dioncophyllaceae and Ancistrocladaceae. *Journal of Experimental Botany* **52**, 2015–2022.
- Bringmann G, Holenz J, Wiesen B, Nugroho BW, Proksch P.** 1997. Dioncophylline A as a growth-retarding agent against the herbivorous insect *Spodoptera littoralis*: structure–activity relationships. *Journal of Natural Products* **60**, 342–347.
- Bringmann G, Kehr C, Dauer U, Gulden K-P, Haller RD, Bar S, Isahakia MA, Robertson SA, Peters K.** 1993. *Ancistrocladus robertsoniorum* produces pure crystalline droserone when wounded. *Planta Medica* **59**, A622–A623.
- Bringmann G, Rischer H, Wohlfarth M, Schlauer J, Assi LA.** 2000. Droserone from cell cultures of *Triphyophyllum peltatum* (Dioncophyllaceae) and its biosynthetic origin. *Phytochemistry* **53**, 339–343.
- Bringmann G, Wohlfarth M, Rischer H, Rückert M, Schlauer J.** 1998. The polyketide folding mode in the biogenesis of isoshinanolone and plumbagin from *Ancistrocladus heyneanus* (Ancistrocladaceae). *Tetrahedron Letters* **39**, 8445–8448.
- Budzianowski J.** 2000. Naphthoquinone glucosides of *Drosera gigantea* from *in vitro* cultures. *Planta Medica* **66**, 667–669.
- Castro FA, Mariani D, Panek AD, Eleutherio EC, Pereira MD.** 2008. Cytotoxicity mechanism of two naphthoquinones (menadione and plumbagin) in *Saccharomyces cerevisiae*. *PLoS One* **3**, e3999.
- Coste A, Selmecki A, Forche A, Diogo D, Bournoux ME, d'Enfert C, Berman J, Sanglard D.** 2007. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. *Eukaryotic Cell* **6**, 1889–1904.
- de Paiva SR, Figueiredo MR, Aragao TV, Kaplan MA.** 2003. Antimicrobial activity *in vitro* of plumbagin isolated from *Plumbago* species. *Memórias do Instituto Oswaldo Cruz* **98**, 959–961.
- DiCosmo F, Misawa M.** 1985. Eliciting secondary metabolism in plant cell cultures. *Trends in Biotechnology* **3**, 318–322.
- Didry NI, Dubreuil L, Trotin F, Pinkas M.** 1998. Antimicrobial activity of aerial parts of *Drosera peltata* Smith on oral bacteria. *Journal of Ethnopharmacology* **60**, 91–96.
- Dinda B, Das S, Hajra A.** 1995. Naphthoquinones from the roots of *Plumbago rosea*. *Indian Journal of Chemistry* **34**, 525–528.
- Dzoyem JP, Tangmouo JG, Lontsi D, Etoa FX, Lohoue PJ.** 2007. *In vitro* antifungal activity of extract and plumbagin from the stem bark of *Diospyros crassiflora* Hiern (Ebenaceae). *Phytotherapy Research* **21**, 671–674.
- Eilenberg H, Pnini-Cohen S, Schuster S, Movtchan A, Zilberstein A.** 2006. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes khasiana*. *Journal of Experimental Botany* **57**, 2775–2784.

- Eilenberg H, Zilberstein A.** 2008. Carnivorous pitcher plants: towards understanding the molecular basis of prey digestion. In: Teixeira da Silva JA, ed. *Floriculture, ornamental and plant biotechnology: advances and topical issues*, Vol. 5, 1st edn. Isleworth, UK: Global Science Books, 287–294.
- Ellison AM, Gotelli NJ.** 2009. Energetics and the evolution of carnivorous plants: Darwin's 'most wonderful plants in the world'. *Journal of Experimental Botany* **60**, 19–42.
- Espinel-Ingroff A, Dawson K, Pfaller M, et al.** 1995. Comparative and collaborative evaluation of standardization of antifungal susceptibility testing for filamentous fungi. *Antimicrobial Agents and Chemotherapy* **39**, 314–319.
- Etkin NL.** 2008. An ethnobotany of Darwin's gardens. *Ethnobotany Research and Applications* **6**, 325–334.
- Gallie DR, Chang SC.** 1997. Signal transduction in the carnivorous plant *Sarracenia purpurea*. Regulation of secretory hydrolase expression during development and in response to resources. *Plant Physiology* **115**, 1461–1471.
- Ganapaty S, Thomas P, Karagianis G, Waterman PG, Brun R.** 2006. Antiprotozoal and cytotoxic naphthalene derivatives from *Diospyros assimilis*. *Phytochemistry* **67**, 1950–1956.
- Hazra B, Sarkar R, Bhattacharyya S, Ghosh PK, Chel G, Dinda B.** 2002. Synthesis of plumbagin derivatives and their inhibitory activities against *Ehrlich ascites carcinoma in vivo* and *Leishmania donovani* promastigotes *in vitro*. *Phytotherapy Research* **16**, 133–137.
- Inbaraj JJ, Chignell CF.** 2004. Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes. *Chemical Research in Toxicology* **17**, 55–62.
- Kaku H, Shibuya N, Xu PA, Aryan AP, Fincher GB.** 1997. Acetylchitooligosaccharides elicit expression of a single (1 → 3)- β -glucanase gene in suspension-cultured cells from barley (*Hordeum vulgare*). *Physiologia Plantarum* **100**, 111–118.
- Kayser O, Kiderlen A, Croft S.** 2003. Natural products as antiparasitic drugs. *Parasitology Research* **90**, S55–S62.
- Khambay BP, Batty D, Jewess PJ, Bateman GL, Hollomon DW.** 2003. Mode of action and pesticidal activity of the natural product dunnione and of some analogues. *Pest Management Science* **59**, 174–182.
- Komaraiah P, Naga Amrutha R, Kavi Kishor P, Ramakrishna S.** 2002. Elicitor enhanced production of plumbagin in suspension cultures of *Plumbago rosea* L. *Enzyme and Microbial Technology* **31**, 634–639.
- Komaraiah P, Ramakrishna SV, Reddanna P, Kavi Kishor PB.** 2003. Enhanced production of plumbagin in immobilized cells of *Plumbago rosea* by elicitation and *in situ* adsorption. *Journal of Biotechnology* **101**, 181–187.
- Kreher B, Lotter H, Cordell GA, Wagner H.** 1988. New furanonaphthoquinones and other constituents of *Tabebuia avellanedae* and their Immunomodulating activities *in vitro*. *Planta Medica* **54**, 562–563.
- Kreher B, Neszmelyi A, Wagner H.** 1990. Naphthoquinones from *Dionaea muscipula*. *Phytochemistry* **29**, 605–606.
- Kubo I, Uchida M, Klocke J.** 1983. An insect ecdysis inhibitor from the African medicinal plant *Plumbago capensis* (Plumbaginaceae); a naturally occurring chitin synthetase inhibitor. *Agricultural and Biological Chemistry* **47**, 911–913.
- Kuchitsu K, Kosaka T, Shiga T, Shibuya N.** 1995. EPR evidence for generation of hydroxyl radical triggered by N-acetylchitooligosaccharide elicitor and a protein phosphate inhibitor in suspension-cultured rice cells. *Protoplasma* **188**, 138–142.
- Lien J, Huang L, Wang J, Teng C, Lee K, Kuo S.** 1996. Synthesis and antiplatelet, antiinflammatory and antiallergic activities of 2,3-disubstituted 1,4-naphthoquinones. *Chemical and Pharmaceutical Bulletin* **44**, 1181–1187.
- Likhitwitayawuid K, Kaewamatawong R, Ruangrunsi N, Krungkrai J.** 1998. Antimalarial naphthoquinones from *Nepenthes thorelii*. *Planta Medica* **64**, 237–241.
- Mandell G, Douglas R, Bennett J.** 2005. *Principles and practice of infectious diseases*. Elsevier.
- Mantyla A, Garnier T, Rautio J, Nevalainen T, Vepsäläinen J, Koskinen A, Croft SL, Jarvinen T.** 2004. Synthesis, *in vitro* evaluation, and antileishmanial activity of water-soluble prodrugs of buparvaquone. *Journal of Medical Chemistry* **47**, 188–195.
- Mehmood Z AI, Mohammad F, Ahmad S.** 1999. Indian medicinal plants: a potential source for anticandidal drugs. *Pharmaceutical Biology* **37**, 237–242.
- Michallet M, Ito JI.** 2009. Approaches to the management of invasive fungal infections in hematologic malignancy and hematopoietic cell transplantation. *Journal of Clinical Oncology* **27**, 3398–3409.
- Miguel del Corral JM, Castro MA, Oliveira AB, Gualberto SA, Cuevas C, San Feliciano A.** 2006. New cytotoxic furoquinones obtained from terpenyl-1,4-naphthoquinones and 1,4-anthracenediones. *Bioorganic and Medicinal Chemistry* **14**, 7231–7240.
- Montoya J, Varela-Ramirez A, Shanmugasundram M, Martinez LE, Primm TP, Aguilera RJ.** 2005. Tandem screening of toxic compounds on GFP-labeled bacteria and cancer cells in microtiter plates. *Biochemical and Biophysical Research Communications* **335**, 367–372.
- Munday R, Smith BL, Munday CM.** 1995. Comparative toxicity of 2-hydroxy-3-alkyl-1,4-naphthoquinones in rats. *Chemico-Biological Interactions* **98**, 185–192.
- Nahalka J, Nahalkova J, Gemeiner P, Blanarik P.** 1998. Elicitation of plumbagin by chitin and its release into the medium in *Drosophyllum lusitanicum* Link. suspension cultures. *Biotechnology Letters* **20**, 841–845.
- Nishizawa Y, Kawakami A, Hibi T, He D, Shibuya N, Minami E.** 1999. Regulation of the chitinase gene expression in suspension cultured rice cells by N-acetylchitooligosaccharides: differences in the signal transduction pathways leading to the activation of elicitor-responsive genes. *Plant Molecular Biology* **39**, 907–914.
- Ogihara K, Yamashira R, Higa M, Yogi S.** 1997. Preparation of naphthoquinone derivatives from plumbagin and their ichthyotoxicity. *Chemistry and Pharmaceutical Bulletin* **45**, 437–445.
- Parimala R, Sachdanandam P.** 1993. Effect of plumbagin on some glucose metabolising enzymes studied in rats in experimental hepatoma. *Molecular and Cellular Biochemistry* **125**, 59–63.

- Patil RS, Ghormade VV, Deshpande MV.** 2000. Chitinolytic enzymes: an exploration. *Enzyme and Microbial Technology* **26**, 473–483.
- Perez-Sacau E, Estevez-Braun A, Ravelo A, Ferro E, Tokuda H, Mukainaka T, Nishino H.** 2003. Inhibitory effects of lapachol derivatives on epstein-barr virus activation. *Bioorganic and Medicinal Chemistry* **11**, 483.
- Pintea AM.** 2007. Quinone biosynthesis. In: Socaciu C, ed. *Food colorants, chemical and functional properties*. CRC Press, 102–104.
- Rajendran L, Suvarnalatha G, Ravishankar G, Venkataraman L.** 1994. Enhancement of anthocyanin production in callus cultures of *Daucus carota* L. under influence of fungal elicitors. *Applied Microbiology and Biotechnology* **42**, 227–231.
- Ramakrishna S, Reddy R, Curtis W, Humphry A.** 1993. Stimulation of solvetivone synthesis in free and immobilized cells of *Hyoscyamus mutucus* by *Rhizoctonia solani* fungal components. *Biotechnology Letters* **15**, 307–310.
- Rischer H, Hamm A, Bringmann G.** 2002. *Nepenthes insignis* uses a C2-portion of the carbon skeleton of L-alanine acquired via its carnivorous organs, to build up the allelochemical plumbagin. *Phytochemistry* **59**, 603–609.
- Sandur SK, Ichikawa H, Sethi G, Ahn KS, Aggarwal BB.** 2006. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappaB activation and NF-kappaB-regulated gene products through modulation of p65 and I-kappaB-alpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. *Journal of Biological Chemistry* **281**, 17023–17033.
- Sankaram A, Srinivasan T, Indulkar A.** 1975. Fungicidal activity of some naturally occurring naphthaquinones. *Pesticide Science* **6**, 165–168.
- Selmecki A, Forche A, Berman J.** 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science* **313**, 367–370.
- Shchekotikhin AE, Buyanov VN, Preobrazhenskaya MN.** 2004. Synthesis of 1-(omega-aminoalkyl)naphthoindoleiones with antiproliferative properties. *Bioorganic and Medicinal Chemistry* **12**, 3923–3930.
- Shin K-S, Lee S, Cha B.** 2007. Antifungal activity of plumbagin purified from leaves of *Nepenthes ventricosa* × *maxima* against phytopathogenic fungi. *Plant Pathology Journal* **23**, 113–115.
- Sidhu GS, Sankaram AVB, Mahmood Ali S.** 1968. Extractives from *Diospyros* species. Part III. New naphthaquinones and naphthols from the heartwood of *Diospyros melanoxylon* Roxb. *Indian Journal of Chemistry* **6**, 681–691.
- Silva T, Camara C, Barbosa T, Soares A, da Cunha L, Pinto A, Vargas M.** 2005. Molluscicidal activity of synthetic lapachol amino and hydrogenated derivatives. *Bioorganic and Medicinal Chemistry* **13**, 193–196.
- Takai R, Hasegawa K, Kaku H, Shibuya N, Minami E.** 2001. Isolation and analysis of expression mechanisms of a rice gene, EL5, which shows structural similarity to ATL family from Arabidopsis, in response to N-acetylchitooligosaccharide elicitor *Plant Science* **160**, 577–583.
- Tandon V, Yadav D, Singh R, Chaturvedi A, Shukla P.** 2005. Synthesis and biological evaluation of novel (l)-α-amino acid methyl ester, heteroalkyl, and aryl substituted 1,4-naphthoquinone derivatives as antifungal and antibacterial agents. *Bioorganic and Medicinal Chemistry Letters* **15**, 5324–5328.
- Tandon VK, Maurya HK, Tripathi A, Shivakeshava GB, Shukla PK, Srivastava P, Panda D.** 2008. 2,3-Disubstituted-1,4-naphthoquinones, 12H-benzo[b]phenothiazine-6,11-diones and related compounds: synthesis and biological evaluation as potential antiproliferative and antifungal agents. *European Journal of Medicinal Chemistry* **44**, 1086–1092.
- Tandon VK, Singh RV, Yadav DB.** 2004. Synthesis and evaluation of novel 1,4-naphthoquinone derivatives as antiviral, antifungal, and anticancer agents. *Bioorganic and Medicinal Chemistry Letters* **14**, 2901–2904.
- Tokunaga T, Takada N, Ueda M.** 2004. Mechanism of antifeedant activity of plumbagin, a compound concerning the chemical defence in carnivorous plants. *Tetrahedron Letters* **45**, 7115–7119.
- Vessey J, Pegg G.** 1973. Chitinase in *Verticillium*. *Transactions of the British Mycological Society* **60**, 133–143.
- Wayne P.** 2002a. *Reference method for broth dilution antifungal susceptibility testing of yeasts*. Approved standard, 2nd edn. NCCLS document M27–A2. National Committee for Clinical Laboratory Standards.
- Wayne P.** 2002b. *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi*. Approved standard. NCCLS document M38–A. National Committee for Clinical Laboratory Standards.
- Yamada A, Shibuya N, Kodama O, Akatsuka T.** 1993. Induction of phytoalexin formation in suspension-cultured rice cells by N-acetylchitooligosaccharides. *Bioscience, Biotechnology and Biochemistry* **57**, 405–409.